

Comparison of the Effects of Infection with *Salmonella enteritidis*, in Combination with an Induced Molt, on Serum Levels of the Acute Phase Protein, α_1 Acid Glycoprotein, in Hens

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ABSTRACT Periods of inflammation due to infection, injury, or malignancy are marked by increases in serum constituents known as acute phase proteins (APP), and these proteins have been used as markers for early stages of disease. Four experiments were performed to examine whether levels in chickens of one such APP, α_1 acid glycoprotein (AGP), would be affected by an infection with *Salmonella enteritidis* (SE) and if the added stress of induced molting via 14-d feed withdrawal would increase these effects. In all experiments but Experiment 1, hens were divided into four equal groups: molted infected, nonmolted infected, molted noninfected, nonmolted noninfected (Experiment 1 lacked this last group). Blood and intestinal samples were collected at various times from the hens and assayed for AGP and SE levels, respectively.

Infection with SE significantly elevated serum AGP levels above those found in the noninfected groups of hens in two of four experiments, whereas in molted infected hens, serum AGP levels were significantly higher than those found in the noninfected counterparts in all four experiments. Comparison of AGP titer between the infected groups of hens revealed that significantly higher SE levels generally did not guarantee significantly higher AGP levels, although when individual values were plotted, a trend was observed toward increasing serum levels concomitant with increasing SE counts. Serum AGP levels show promise as a method to detect infection problems in hens, especially when the severity of the infection is exacerbated by stress situations. However, more work is needed to determine what other factors may elevate serum AGP levels and potentially confound the picture.

(Key words: acute phase protein, inflammation, enteric infection, *Salmonella enteritidis*)

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INTRODUCTION

Acute phase proteins (APP) compose a large and varied group of serum proteins. Synthesized in the liver parenchyma, APP are released into the bloodstream by a variety of stimuli including inflammation (Thomas and Schreiber, 1985; Fleck, 1989; Jamieson et al., 1992), bacterial infection (Morley and Kushner 1982; Pfeffer, 1988; Pfeffer and Rogers, 1989), endotoxin exposure (Takahashi et al., 1994), neoplasia (Ganz et al., 1983), and injury (Neuhaus et al., 1966; Bosanquet et al., 1976; Koj and Gordon 1985). The biological functions of APP vary widely: protease inhibitors, enzymes, transport proteins, coagulation proteins, and modulators of the immune response, but all APP appear to play a role in the restoration of homeostasis after tissue necrosis or injury (Koj and Gordon, 1985; Kushner and Mackiewicz, 1987; Takahashi et al., 1994). The presence of inflammatory cytokines such as interleu-

kin-1, interleukin-6, and tumor necrosis factor amplifies the synthesis of APP (Klasing, 1984; Marinkovic et al., 1989; Baumann and Gauldie 1990, 1994).

Although the APP response pattern to various stimuli may differ, changes in serum APP concentrations are generally regarded as being sensitive, although nonspecific, indicators of inflammation. Some APP such as C-reactive protein and serum amyloid A in humans (Pepys, 1979) and haptoglobin in ruminants (Skinner et al., 1991) are considered major APP and have been used to detect and monitor infection, inflammatory disease, and cancer.

Induced molting is a prevalent management tool used by the layer industry in the US to give hens a rest after an extensive egg lay. However, previous studies in our laboratory (Holt and Porter, 1992; Holt, 1993, 1995; Holt et al., 1995) have shown that inducing a molt through chronic feed removal increased the potential for problems with SE infection, including increased susceptibility to infection, more SE shed, and rapid horizontal transmission of SE to noninfected birds. In some of the studies, it was noted that early in the infection, there was dramati-

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Abbreviation Key: AGP = α_1 acid glycoprotein; APP = acute phase protein; SPF = specific pathogen free.

cally increased inflammation in the intestinal tract of molted infected birds, primarily in the cecum (Holt and Porter, 1992; Macri et al., 1997), which was generally not observed of nonmolted infected birds. Elevated levels of the inflammatory cytokine tumor necrosis factor were found in the intestinal tract of these birds (Arnold and Holt, 1996). As noted above, such inflammation should elicit a significant acute phase response detectable with appropriate assay systems. A detection kit specific for the chicken APP α_1 acid glycoprotein (AGP) has recently become available commercially and has been used in several preliminary studies to assess inflammation in chickens (Fischbach et al., 1996; Goclan et al., 1996; Laudert et al., 1996). A simple method to assess the inflammation status of a flock undergoing molt may be a valuable tool for producers to detect potential problems, such as SE infection, in their flocks during this period of high susceptibility. The objective of the current study was to compare serum levels of AGP in hens infected with SE to those of noninfected hens and determine whether these levels are affected by the added stress of an induced molt.

MATERIALS AND METHODS

Experimental Design

All experiments were conducted in climate-controlled biocontainment buildings at the Southeast Poultry Research Laboratory. Hens were fed antibiotic-free layer ration until commencement of molt at which time feed was withdrawn from the molt group for 14 d followed by grower ration for the remainder of the experiment. On Day 4 of molt, the infected groups of hens (molted and nonmolted) were challenged with 1×10^7 cells of a nalidixic-acid-resistant SE (Experiments 1 and 2) or a rifampicin-resistant SE (Experiments 3 and 4). Infected and noninfected hens were housed in separate rooms in the same building. The hens were periodically bled from the brachial wing vein on the days noted for each experiment, and the serum was collected from each sample and frozen until assayed for AGP levels as noted below. The hens were also sampled for SE shedding by injecting 0.5 mL of 5% pilocarpine intraperitoneally and collecting the alimentary secretions over the next h (Holt and Porter, 1992). Serial 10-fold dilutions were made of the secretions and the dilutions, along with the undiluted sample, and were spread-plated onto brilliant green agar containing 20 $\mu\text{g/mL}$ each of nalidixic acid and novobiocin (Experiments 1 and 2) or 100 $\mu\text{g/mL}$ rifampicin (Experiments 3 and 4). One milliliter of each sample was also added to 9 mL tetrathionate brilliant green. The plates and tetrathionate tubes were incubated for 24 h at 37 C. For those samples with no detectable SE on plates, the tetrathionate enrichments were then streaked onto the appropriate plates and, after further incubation at 37 C, the plates were examined for the presence of SE. Samples with no

growth in the direct plating but positive in the tetrathionate enrichment were given an arbitrary count of 9 (1 below the theoretical detection limit), and samples with no growth in the direct plating or the tetrathionate enrichment were given a count of 0.

Experiment 1. Twenty-six Single Comb White Leghorn hens, 88 wk old, retired from the laboratory specific pathogen-free (SPF) flock, were placed into individual layer cages in two separate climate-controlled biocontainment rooms, 18 hens in Room 1 and eight hens in Room 2, and were allowed to acclimate for 1 wk. Feed was then removed from nine hens in Room 1 (molted infected) and the eight hens in Room 2 (molted noninfected). The remaining hens in Room 1 remained on feed throughout the experiment (nonmolted infected). On Day 4 of feed removal, the hens in Room 1 were challenged, and all hens were bled and sampled for SE shedding 3 and 9 d later.

Experiment 2. Thirty-two SPF Single Comb White Leghorn hens, 85 wk old, were placed into individual layer cages in two separate rooms, 16 hens in Room 1 and 16 hens in Room 2, and were allowed to acclimate for 1 wk. Feed was then removed from eight hens in Room 1 (molted infected) and eight hens in Room 2 (molted noninfected), whereas the remaining hens in Room 1 (nonmolted infected) and Room 2 (nonmolted noninfected) remained on feed throughout the experiment. On Day 4 of feed removal, the hens in Room 1 were challenged, and all hens were bled and sampled for SE shedding 3 d later.

Experiment 3. Thirty-two SPF Single Comb White Leghorn hens, 58 wk old, were placed into individual layer cages in two separate rooms, 16 hens in Room 1 and 16 hens in Room 2, and were allowed to acclimate for 1 wk. Feed was then removed from eight hens in Room 1 (molted infected) and eight hens in Room 2 (molted noninfected), whereas the remaining hens in Room 1 (nonmolted infected) and Room 2 (nonmolted noninfected) remained on feed throughout the experiment. The hens in Room 1 were challenged at Day 4 of feed removal and bled on molt Days 1, 4, 7 (Day 3 postchallenge), 12 (Day 8 postchallenge), and 14 (Day 10 postchallenge).

Experiment 4. Thirty-two White Leghorn hens (Hy-Line W36), 56 wk old, from a local commercial facility were placed into individual layer cages in two separate rooms, 16 hens in Room 1 and 16 hens in Room 2, and were allowed to acclimate for 1 wk. Feed was then removed from eight hens in Room 1 and eight hens in Room 2. The hens in Room 1 were challenged at Day 4 of feed removal and bled on molt Days 2, 5 (Day 1 postchallenge), 7 (Day 3 postchallenge), 9 (Day 5 postchallenge), 11 (Day 7 postchallenge), 13 (Day 9 postchallenge) and 14 (Day 10 postchallenge).

AGP Assay. Serum levels of AGP were determined using a commercially available radial immunodiffusion tray.² The trays contain agarose impregnated with anti-sera specific for chicken AGP. Ten wells are cut in the agarose for application of sample. Control samples containing 250 $\mu\text{g/mL}$ and 1,000 $\mu\text{g/mL}$ AGP were supplied

²Cardiotech Services, Inc., Louisville, KY.

TABLE 1. Serum α_1 -acid glycoprotein (AGP) levels as a function of SE infection and molt status—Experiments 1 and 2

Hen group	Day Postchallenge ¹			
	Day 3		Day 9	
	AGP ($\mu\text{g/mL}$)	SE (\log_{10})	AGP ($\mu\text{g/mL}$)	SE (\log_{10})
Experiment 1				
Molted infected	1,238 \pm 147 ^a	5.98 \pm 0.49	1276 \pm 117 ^a	7.12 \pm 0.35 ^a
Nonmolted infected	919 \pm 103 ^a	4.84 \pm 0.44	1046 \pm 156 ^{ab}	5.04 \pm 0.36 ^b
Molted noninfected	596 \pm 53 ^b	0	843 \pm 87 ^{b+}	0
Experiment 2				
Molted infected	864 \pm 126 ^a	5.8 \pm 0.58 ^a		
Nonmolted infected	648 \pm 158 ^{ab}	2.8 \pm 0.57 ^b		
Molted noninfected	209 \pm 64 ^b	0		
Nonmolted noninfected	164 \pm 40 ^b	0		

^{a,b}Values in a column not followed by a common letter, in each experiment, are different at $P < 0.05$.

¹Results represent the mean \pm SEM of serum AGP and intestinal SE levels at various times post-SE challenge.

with each kit, and 5 μL of each control was administered to an individual well in each tray, the results of which provided the points for generating a standard curve. Five microliters of test serum was added to the other eight wells, and the trays were placed into a moisture chamber and incubated overnight at 25 C. The presence of AGP was indicated by the formation of a ring around the well (Figure 1) resulting from the interaction of the AGP migrating into the agarose and the anti-AGP antibodies already present in the matrix. Concentration of AGP directly correlates with the diameter of the ring—higher concentrations means larger ring diameters. Ring diameters of the two control AGP samples and the values were plotted on semi-logarithmic paper with AGP concentration on the horizontal axis and ring diameter on the vertical axis. A line was drawn between the two points that served as reference curve for determining the concentration in the remaining eight samples.

Statistics. SE counts from intestinal samples were transformed to \log_{10} colony-forming units. Analysis of variance was calculated on these transformed counts using Instat Software³ and on the serum AGP levels with significance determined at $P < 0.05$.

RESULTS AND DISCUSSION

Infection has been shown previously to elevate serum APP in humans (Kushner and Mackiewicz, 1987), sheep (Pfeffer, 1988; Pfeffer and Rogers, 1989), cattle (Godson et al., 1995; Deignan et al., 2000), and chickens (Fischbach et al., 1996; Goclan et al., 1996; Laudert et al., 1996), and similar results were observed in the first two experiments. In Experiment 1, hens from the two challenged groups shed similar levels of SE at Day 3 postchallenge, and no significant differences in serum AGP levels were observed between these two groups, whereas both infected groups exhibited significantly higher AGP levels than the molted noninfected group (Table 1). By Day 9

postchallenge, the molted infected group of hens shed significantly more SE than the nonmolted infected hens, but serum AGP levels, although numerically higher (230 $\mu\text{g/mL}$) in molted infected hens, were not significantly different from levels in the nonmolted infected group. However, AGP levels in the molted infected hens were significantly higher than those of molted noninfected hens. In Experiment 2 (Table 1), similar results were observed in which both of the infected groups of hens had significantly higher serum AGP levels than the molted noninfected or nonmolted noninfected hens. The molted infected hens shed significantly more SE than the nonmolted infected hens, but serum AGP levels between the two groups were not significantly different. Deignan et al. (2000) observed that elevated levels of the serum APP haptoglobin in calves increases following challenge with *Salmonella*, and the degree of increase is correlated with severity of disease. Comparable effects were observed in cows with mastitis (Salonen et al., 1996) and viral respiratory disease (Godson et al., 1995). However,

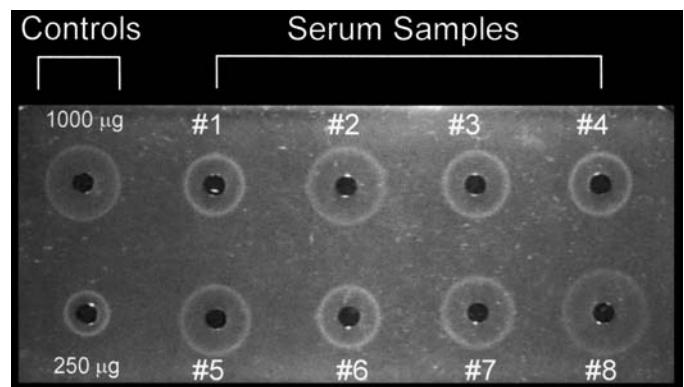


FIGURE 1. Photograph of α_1 acid glycoprotein (AGP) radial immunodiffusion plate showing precipitin band for the 1,000 $\mu\text{g/mL}$ and 250 $\mu\text{g/mL}$ controls and eight serum samples from infected hens. Five microliters of sample was added to each well, and then the trays were incubated in a humidified chamber for 24 h at room temperature. The diameters of the bands were then measured, and the serum AGP concentrations of the unknown samples were extrapolated from a line derived by plotting the two control readings.

³GraphPad Software, San Diego, CA.

TABLE 2. Serum α_1 -acid glycoprotein (AGP) levels as a function of SE infection and molt status over time—Experiment 3

Hen group	AGP ($\mu\text{g/mL}$) at day Postchallenge ¹			
	Day 1	Day 3	Day 8	Day 10
Molted infected	974 \pm 121 ^a	1,111 \pm 160 ^a	1,436 \pm 123 ^a	1,126 \pm 138 ^a
Nonmolted infected	836 \pm 86 ^a	1,039 \pm 121 ^a	923 \pm 118 ^b	821 \pm 56 ^{ab}
Molted noninfected	897 \pm 44 ^a	889 \pm 80 ^a	791 \pm 56 ^b	688 \pm 99 ^b
Nonmolted noninfected	851 \pm 70 ^a	855 \pm 64 ^a	757 \pm 57 ^b	611 \pm 138 ^b

^{a,b}Values in a column not followed by a common letter are different at $P < 0.05$.

¹Results represent the mean \pm SEM of serum AGP levels at various times post-SE challenge.

in the current study, serum AGP concentrations appeared to be less affected by intestinal bacterial loads than by the actual presence of SE. The infected hens, as a group, showed significantly elevated serum AGP levels compared with their noninfected counterparts, but between the two infected groups of hens, significantly higher levels of SE did not concomitantly result in a significantly higher serum AGP. Other factors may be involved in the AGP elevation. Minimal differences were observed between the two noninfected groups of hens, indicating that molting alone does not elevate serum AGP levels.

Two further experiments were undertaken to determine more closely the time frame for AGP synthesis by examining more time points during the infection cycle. In Experiment 3, the molted infected hens shed 6.8 log₁₀ SE compared with 3.1 log₁₀ SE in the nonmolted infected hens ($P < 0.001$) and 6.5 log₁₀ SE compared with 2.6 log₁₀ SE ($P < 0.01$) in the nonmolted infected hens at Days 4 and 10, respectively. No significant differences in serum AGP levels were observed between the four groups of hens in Experiment 3 at Days 1 and 3 postchallenge (Table 2), but by Day 8, the serum AGP levels were significantly higher in the molted infected hens compared with the other three treatment groups and remained significantly higher than the two noninfected groups at Day 10. The AGP titers in the nonmolted infected hens, although elevated, were not significantly different from those in the two noninfected groups, possibly due to the unusually high serum AGP levels found in these latter two groups of birds throughout the experiment.

In a repeat experiment, hens were obtained from a commercial operation in order to determine whether

serum AGP responses to SE infection and molting differed between SPF hens and hens used in the field. Similar to what was observed previously in this and other studies (Holt and Porter, 1992), the molted infected hens shed significantly higher levels of SE than nonmolted infected hens at Day 4 (6.5 log₁₀ SE vs. 1.5 log₁₀ SE) and Day 10 (3 log₁₀ SE vs. 0.3 log₁₀ SE). Serum AGP levels were equivalent in the four groups at Day -3 postchallenge (Day 1 of molt, data not shown). However, significantly higher levels of AGP were detected in the sera of the two infected groups of hens at Days 1 and 3 postchallenge compared with serum from the two noninfected groups (Table 3).

Although the molted infected hens shed approximately 5 logs more SE than their nonmolted counterparts at Day 4 postchallenge, Day 3 levels of serum AGP were not significantly different between the two groups. However, when serum AGP values are plotted relative to the number of SE shed (Figure 2), the trend toward increasing SE numbers resulted in concomitantly increasing serum AGP titers, similar to what was observed by Deignan et al. (2000) for serum haptoglobin levels in calves infected with *Salmonella*. The concentrations of serum AGP remained elevated in the molted infected hens and were significantly higher than those found in the other three groups on Days 5, 7, and 10 postchallenge. On these same days, AGP levels in serum from nonmolted infected hens was significantly higher than that found in nonmolted noninfected hens but not in molted noninfected hens.

The results from the current study indicate that serum AGP levels can be an effective and rapid indicator of infection, including that by SE. Similar to C reactive protein and serum amyloid A in humans (Pepys, 1979)

TABLE 3. Serum α_1 -acid glycoprotein (AGP) levels as a function of SE infection and molt status over time—Experiment 4

	AGP ($\mu\text{g/mL}$) at day Postchallenge ¹				
	Day 1	Day 3	Day 5	Day 7	Day 10
Molted infected	630 \pm 83 ^{a2}	876 \pm 115 ^a	1,243 \pm 140 ^a	1,268 \pm 128 ^a	1,057 \pm 93 ^a
Nonmolted infected	641 \pm 34 ^a	738 \pm 86 ^a	791 \pm 67 ^b	703 \pm 37 ^b	714 \pm 51 ^b
Molted noninfected	312 \pm 41 ^b	312 \pm 41 ^b	491 \pm 53 ^{bc}	534 \pm 55 ^{bc}	453 \pm 41 ^{bc}
Nonmolted noninfected	286 \pm 42 ^b	286 \pm 42 ^b	293 \pm 58 ^c	343 \pm 45 ^c	311 \pm 75 ^c

^{a,b}Values in a column not followed by a common letter are different at $P < 0.05$.

¹Results represent the mean \pm SEM of serum AGP levels at various times post-SE challenge.

²[AUTH QUERY: Please complete or delete footnote in column 2]

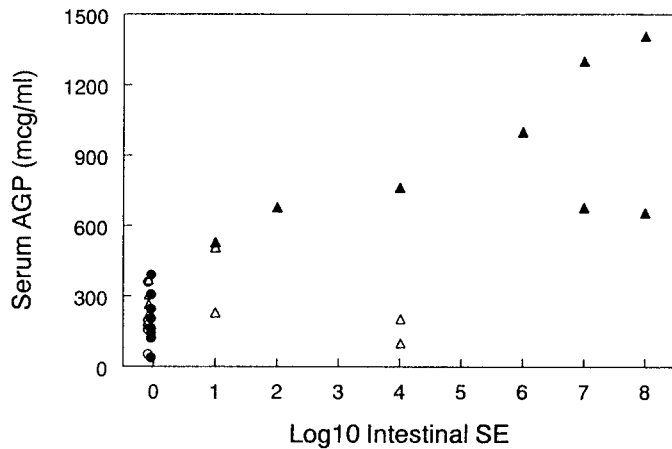


FIGURE 2. Scatterplot of the serum α_1 acid glycoprotein (AGP) levels vs. intestinal SE concentrations in molted infected (closed triangles), nonmolted infected (open triangles), molted noninfected (closed circles), and nonmolted noninfected (open circles) hens at Day 3 postchallenge in Experiment 4.

and haptoglobin in cattle (Skinner et al., 1991), serum AGP levels may have utility for following the infection status of a flock. Exposure of the flock to a potential stress situation, such as molting, may exacerbate the infection and therefore may elevate the serum AGP levels further (Tables 2 and 3). However, previous studies have also shown that many factors can elevate serum APP levels (Thomas and Schreiber, 1985; Neuhaus et al., 1966), and it remains to be determined what effects stimuli such as vaccination, trauma from cagemates, and house ammonia levels may have on the baseline levels of AGP in a flock. Those findings may ultimately limit the utility of such an assay system for use in the field.

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